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Vaccination with Allergen-IL-18 Fusion DNA Protects Against, and Reverses Established, Airway Hyperreactivity in a Murine Asthma Model

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Vaccination with naked DNA encoding a specific allergen has been shown previously to prevent, but not reverse, the development of allergen-induced airway hyperresponsiveness (AHR). To enhance the effectiveness of DNA vaccine therapies and make possible the treatment of established AHR, we developed a DNA vaccination plasmid containing OVA cDNA fused to IL-18 cDNA. Vaccination of naive mice either with this fusion DNA construct or with an OVA cDNA-containing plasmid protected the mice from the subsequent induction of AHR. Protection from AHR correlated with increased IFN-γ production and reduced OVA-specific IgE production. The protection appeared to be mediated by IFN-γ and CD8+ cells because treatment of mice with neutralizing anti-IFN-γ mAb or with depleting anti-CD8 mAb abolished the protective effect. Moreover, vaccination of mice with preexisting AHR with the OVA-IL-18 fusion DNA, but not with the OVA cDNA plasmid, reversed established AHR, reduced allergen-specific IL-4, and increased allergen-specific IFN-γ production. Thus, combining IL-18 cDNA with OVA cDNA resulted in a vaccine construct that protected against the development of AHR, and that was unique among cDNA constructs in its capacity to reverse established AHR. The Journal of Immunology, 2001, 166: 959–965.

The prevalence of allergic asthma has dramatically increased over the last two decades and is a major public health concern (1, 2). Asthma is characterized by airway hyperresponsiveness (AHR) to a variety of specific and nonspecific stimuli, chronic pulmonary inflammation with eosinophilia, excessive mucus production, and high serum IgE levels. The pathology in asthma results from excessive production of IL-4, IL-5, and IL-13 by CD4+ Th2 cells (3, 4). Current treatments for asthma are not satisfactory, and disease prevention is not possible. Given the high prevalence of this disease, improved and more effective therapeutic strategies are needed.

One therapeutic approach for asthma might be to use DNA-based immunization to manipulate the immune system, and to alter the underlying Th2-biased allergic response in an allergen-specific manner. Vaccination with allergen in the form of naked plasmid cDNA stimulates allergen-specific immune responses with a Th1 bias and amplifies the expansion of CD4+ T cells producing IFN-γ and of cytotoxic CD8+ T cells (5–9). The key feature of this strategy is that injection of plasmid DNA encoding a specific Ag produces an allergen-specific protective immune response that should be reinforced by natural exposure to the allergen, thus conferring long-lasting protection (10, 11). Previous studies with DNA immunization strategies demonstrated its success in preventing the development of Ag-specific IgE synthesis and AHR (6, 12, 13). However, although the genetic vaccination approach has succeeded in preventing allergic diseases and has been effective in models of infectious disease (14–16), cancer (17–19), and autoimmune disease (20), the efficacy of DNA vaccination has varied widely, and successful reversal of ongoing AHR with DNA vaccination has not been reported. Thus, improvement of gene vaccination methodologies is required for successful clinical application of DNA vaccination to symptomatic patients with allergic asthma.

To enhance the effectiveness of DNA vaccination and potentially treat patients with ongoing AHR, we constructed a DNA vaccination plasmid containing cDNA for a prototypic allergen, OVA, fused to the cDNA of a potent immune modulating cytokine, IL-18. This approach is based on the fact that IL-18, a product of activated macrophages and Kupffer cells (21–25), is very powerful in driving the production of Th1 cytokine synthesis in naive and memory T cells (26, 27). Furthermore, the rationale for the approach of fusing IL-18 cDNA with OVA cDNA was based on our previous findings with protein-based vaccines (28). In those experiments, an OVA-IL-12 fusion protein increased the immunogenicity of OVA, focused the effects of IL-12 on Ag-specific immune cells, and minimized the Ag-nonspecific effects of IL-12. In this study, we examined the efficacy of the OVA-IL-18 DNA fusion construct vector in a murine model of asthma and compared its efficacy with that of OVA DNA, IL-18 DNA, or a mixture of...
OVA DNA and IL-18 DNA on separate plasmids. We found that the OVA-IL-18 cDNA construct was more potent in protecting against the development of AHR than the other constructs, and that the OVA-IL-18 cDNA construct was unique in its capacity among cDNA constructs to effectively reverse established AHR. These studies suggest that modification of vaccination plasmids with IL-18 cDNA can greatly enhance the immunogenicity of Ag cDNA constructs, and generate constructs that may be clinically effective in treating patients with established allergic pulmonary disease.

Materials and Methods

BALB/cByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were used between 6 and 10 wk of age and were age and sex matched within each experiment. The Stanford University Committee on Animal Welfare approved all animal protocols.

mAbs and reagents

mAbs were purified from ascites fluid by ammonium sulfate precipitation and ion-exchange chromatography. We used the following hybridomas: R462A (anti-IFN-γ; obtained from American Type Culture Collection, Manassas, VA); XMGL1.2 (anti-IFN-γ; provided by Dr. T. Mosmann, University of Alberta, Edmonton, Canada); BV4D-1D11 (anti-IL-4) and BV6D-24G2 (anti-IL-4); obtained from Dr. M. Howard, DNAX Research Institute, Palo Alto, CA); 53.6.7 (anti-CD8; provided by Dr. Irving Weissman, Stanford University); and EM95 (rat anti-mouse IgE; generated by Z. Eshhar, Weizman Institute, Rehovot, Israel and provided by Dr. R. Coffman, DNAX Research Institute). Anti-OVA mAbs and biotinylated anti-ova mAb were produced as described previously (28).

DNA constructs

A series of plasmids expressing OVA fused to various cytokines was produced in our laboratory and has been described previously (9). OVA-DNA plasmids, expressing OVA-IL-4, was digested with XhoI and BamHI to excise the IL-4 portion of the insert. The remainder of the plasmid was ligated to a similarly digested PCR product encoding mature turine IL-18 (Fig. 1A). This sequence was isolated by PCR amplification of cDNA produced from RNA of C57 mouse splenocytes activated with Con A. The forward PCR primer, which incorporated an XhoI site, was 5’-CATGCC GAACATCTTGGGCGACTTAC-3’. The reverse PCR primer, which incorporated a BamHI site, a stop codon, and a hexahistidine tag, was 5’-GTTAGATCTCATATGGTATGCGTGATGATCGTATTGGT AAGTGTAGT-3’. The ligated plasmid (Fig. 1A) was electroporated into Escherichia coli and purified from a large-scale culture by alkaline lysis and CsCl density gradient centrifugation (29). This preparation was then sequenced to verify the correct insertion and correct sequence of the IL-18 fusion construct. Control plasmids, expressing either OVA alone or an irrelevant sequence (single-chain Fv), have also been described previously (9). Finally, a plasmid expressing IL-18 alone was produced for this study. The IL-18 sequence was moved into another vector, pTCAE 5.3, by PCR amplification that added restriction sites (see below). Within 5 days of the last challenge, blood was taken, mice were sacrificed, lungs were removed and fixed, and splenocytes were isolated for in vitro culture.

Ag specificity test

In experiments to determine whether the effects of the different DNA constructs on the immune response of BALB/c mice were Ag specific, mice were immunized with the different OVA DNA constructs i.m. (see above). One week later, the mice were immunized in the footpads either with the relevant Ag, OVA (200 μg/mouse), or an irrelevant Ag, keyhole limpet hemocyanin (KLH, 100 μg/mouse), each emulsified in IFA. After 7 days the mice were sacrificed, and lymphocytes were isolated from the draining lymph nodes (LN) for in vitro culture.

Treatment of mice with anti-cytokine and depletion mAb

BALB/c mice were injected i.p. with 1 mg of mAb XMG1.2 (for IFN-γ depletion), 200 μg of anti-IL-4 mAb BVD4-1D11 (anti-IL-4) and BVD6-24G2 (anti-IL-4); obtained from Dr. M. Howard, DNAX Research Institute, Palo Alto, CA); 53.6.7 (anti-CD8; provided by Dr. Irving Weissman, Stanford University); and EM95 (rat anti-mouse IgE; generated by Z. Eshhar, Weizman Institute, Rehovot, Israel and provided by Dr. R. Coffman, DNAX Research Institute). Anti-OVA mAbs and biotinylated anti-OVA mAb were used as described previously (28).

Reversal of established AHR (Fig. 1D, bottom)

To investigate whether DNA immunization can reverse established AHR rather than inhibit the development of AHR, BALB/c mice were first sensitized with OVA before vaccination with the DNA plasmids. OVA (50 μg) adsorbed to alum was administered i.p. once on day 0. OVA (50 μg) in 50 μl PBS was administered i.n. on days 8 and 9. On days 10 and 25 the different DNA constructs were injected i.m. in the quadriceps muscles (100 μg in 100 μl 0.9% NaCl). On day 39 the mice were boosted again with OVA i.n., and AHR was measured 1 day later (day 40). Mice were sacrificed, and bronchial LN cells were isolated for in vitro culture within 5 days of the last OVA challenge.

Measurement of airway responsiveness

Airway responsiveness was assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole body plethysmograph (model PLY 3211; Buxco Electronics, Troy, NY). Pulmonary airflow obstruction was measured by P2enh using the following formula: P2enh = (Te/RT - 1) (PEF/PIF), where P2enh = enhanced pause (dimensionless), Te = expiratory time, RT = relaxation time, PEF = peak expiratory flow (ml/s), and PIF = peak inspiratory flow (ml/s) (31). Enhanced pause (P2enh) minute volume, tidal volume, and breathing frequency were obtained from chamber pressure, measured with a transducer (model TRD5100) connected to pneumotraphic (model MAX2270), and analyzed by system XA software (model SFT 1810). Measurements of methacholine responsiveness were obtained by exposing mice for 2 min to aerosolized 0.9% NaCl produced by a sonicator (Portable Ultrasonic, 5500D; DeVilbiss Health Care, Sommerset, PA), followed by incremental doses (2.5–20 mg/ml) of aerosolized 0.9% NaCl in 0.9% NaCl). On day 39 the mice were boosted again with OVA i.n., and AHR was measured 1 day later (day 40). Mice were sacrificed, and bronchial LN cells were isolated for in vitro culture within 5 days of the last OVA challenge.

OVA-specific IgE assay

Mice were bled at the time of sacrifice, and OVA-specific IgE was determined using a modified Ag-specific ELISA. Plates were coated overnight with rat anti-mouse IgE mAb EM95 (5.0 μg/ml). After washing and blocking, samples were applied and incubated overnight. Plates were washed and biotinylated OVA (10 μg/ml) was added. Two hours later, plates were washed and HRP-conjugated streptavidin (Southern Biotechnology Associates, Birmingham, AL) was added. Plates were developed with o-phenylenediamine substrate, and the OD was determined at 492 nm. Serum from mice hyperimmunized with OVA in alum was standardized for IgE levels against an anti-OVA IgE mAb provided by E. Gelfand (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). This serum was used as a standard in the OVA-specific IgE ELISA.

Restimulation of spleen and LN cells in vitro

Spleens or bronchial LN cells were removed, depleted of resting B cells by adherence to goat anti-mouse Ig-coated plates (32), and 4 × 10⁶ cells were restimulated in vitro with OVA (100 μg/ml) or KLH (10 μg/ml). Cells were cultured in 96-well microtiter plates in 150 μl medium. Supernatants were harvested after 4 days for determination of IL-4 and IFN-γ levels. Cytokine content in each sample was measured in triplicate by ELISA.
IL-18 assays

IL-18 activity was examined by ELISA and by bioassay. Plasmids expressing IL-18, OVA-IL-18 or, as a control, OVA-IL-4, were transfected into COS-7 cells using DEAE-dextran in a standard method (29). Supernatants from the transfected cells were harvested after 3 days and stored at 4°C. IL-18 ELISA was performed using reagents and protocols from PharMin. To determine IL-18 bioactivity of the OVA-IL-18 fusion construct, the recombinant protein was tested for induction of IFN-γ production by a murine Th1 cell line, DOH2. The murine Th1 cell line, DOH2, was produced and maintained as described previously (33). DOH2 cells were re-suspended at 5 X 10⁶ per ml in DMEM with 10% FBS. Cell suspension (100 μl per well of a microtiter plate) was plated, along with 100 μl of media or COS-7 supernatant at dilutions of 1:2, 1:4, 1:8, or 1:16. The cells were incubated at 37°C for 48 h, then supernatants were harvested from each well and tested for IFN-γ production by ELISA as described (9).

Cytokine ELISA

ELISA for IL-4 and IFN-γ were performed as previously described (32). The mAbs used were as follows, listed by capture/biotinylated detection mAb: IFN-γ, R4-6A2/XMG1.2; IL-4, 1B11/BVD6-24G2. A standard curve using recombinant cytokine in 1:2 dilutions from 20 to 0.156 ng/ml for IFN-γ, or from 500 to 7.5 pg/ml for IL-4, was used for quantitation.

Collection of bronchoalveolar lavage (BAL) fluid

Animals were sacrificed by CO₂ asphyxiation. The trachea was cannulated, and the lungs were lavaged four times with 300 μl of 1% BSA in 1× PBS. Cells in the lavage fluid were then counted using a hemocytometer, and BAL cell differentials were determined on slide preparations stained with HaeIII stain (Lide Laboratories, Florissant, MO). At least 200 cells were differentiated by light microscopy based on conventional morphogenetic criteria.

Results

IL-18 bioactivity of fusion construct

To test the bioactivity of the OVA-IL-18 and the IL-18 plasmids, COS-7 cells were transiently transfected with plasmid DNA, and the cells were cultured for 4 d. We then examined the supernatants from these cells cultures for IL-18 bioactivity, via the ability to induce IFN-γ production in a Th1 cell line, and for IL-18 protein content by ELISA. Fig. 1B shows that supernatant from OVA-IL-18-transfected COS-7 cells induced IFN-γ production from the established Th1 cell line, DOH2, whereas medium or supernatant from control OVA-IL-4-transfected cells did not, indicating that protein produced from the IL-18 plasmid had biological activity. The COS-7 supernatants generated from both the IL-18 and OVA-IL-18 plasmids had comparable IL-18 activity (ranges of 0.3–6.3 ng/ml in independent transfection experiments). In addition, ELISA of the COS-7 supernatants indicated that both the IL-18 and the OVA-IL-18 constructs induced the production of IL-18 protein (Fig. 1C).

Inhibition of AHR by vaccination with different DNA vectors

Having established that the IL-18 and OVA-IL-18 fusion constructs had significant IL-18 bioactivity, the plasmids were next tested in vivo for their ability to inhibit AHR in a murine asthma model. BALB/c mice were vaccinated i.m. with irrelevant DNA, OVA DNA, IL-18 DNA, or the OVA-IL-18 DNA fusion construct. The mice were then sensitized for AHR with i.p. and i.n. administrations of OVA (Fig. 1D, prevention model). Fig. 24 demonstrates that sensitization of mice with OVA resulted in the development of significant AHR when the mice were challenged with methacholine. Vaccination of mice with the OVA-IL-18 DNA fusion construct dramatically inhibited the development of AHR. Vaccination with OVA DNA, OVA DNA + IL-18 DNA, or IL-18 DNA alone also inhibited development of AHR, although to a lesser extent. Injection of irrelevant DNA had no effect on the OVA-induced AHR.

Effects of DNA vaccination on cytokine production

A known property of both IL-18 and DNA vaccination in general is the ability to induce IFN-γ production in vivo. To determine whether the reduced AHR in mice vaccinated with the OVA-IL-18 plasmid correlated with alteration of cytokine profiles in CD4⁺ T cells, mice were sacrificed after measurement of airway reactivity. Spleen cells were removed and stimulated with OVA in vitro. Fig. 2B shows that DNA vaccination significantly increased OVA-specific IFN-γ production in OVA-immunized mice. The strongest IFN-γ increase was induced by the OVA-IL-18 DNA fusion construct. The increase of IFN-γ production was comparable in the groups that received either OVA DNA or IL-18 DNA alone, and was slightly higher after injection of the mixture of OVA DNA and IL-18 DNA. Although vaccination with OVA DNA, IL-18 DNA, or a mixture of OVA DNA and IL-18 DNA also induced IL-4 production, the OVA-IL-18 DNA fusion construct did not significantly reduce IL-4 levels in splenic T cells from OVA-immunized mice (data not shown). (Bronchial LN T cells were examined for cytokine production in subsequent experiments, see Fig. 5C.) Irrelevant DNA had no significant effect on IFN-γ or IL-4 production.

Inhibition of IgE synthesis by DNA vaccination

We also analyzed the levels of anti-OVA IgE Ab responses in serum collected from these mice. OVA-specific IgE was very high in OVA-immunized BALB/c mice (Fig. 2C). Vaccination with the different DNA vectors before immunization with OVA significantly reduced the level of OVA-specific IgE. The inhibitory effect on IgE production was strongest with the OVA-IL-18 fusion construct and did not differ significantly between OVA DNA, IL-18 DNA, or the mixture of OVA DNA and IL-18 DNA. In contrast, irrelevant DNA had little effect on IgE production.

Specificity of DNA vaccination

To test whether the effects of immunization with OVA-IL-18 DNA were Ag specific, we boosted DNA-vaccinated mice after 1 wk with either the relevant protein (OVA) or with an irrelevant protein, KLH. After 7 days, spleens were removed and splenocytes were cultured with the Ag used for boosting. Fig. 3, left, shows that in OVA protein-boosted mice, the increase in IFN-γ was most notable in the group that received the OVA-IL-18 DNA fusion construct. In contrast, in mice boosted with the irrelevant Ag KLH, IFN-γ production was not increased by vaccination with OVA-IL-18 DNA, and was similar in all groups receiving the various OVA DNA constructs (Fig. 3, right). These results indicated that vaccination with the OVA-IL-18
DNA was dependent upon both IFN-γ effect on AHR (data not shown). Thus, inhibition of AHR by OVA-IL-18 Ab to CD8+ OVA with either the anti-IFN-γ or the anti-CD8 mAb also restored AHR, although to a lesser extent than that seen with CD8 depletion. Treatment of mice sensitized and challenged with OVA protein by administering OVA i.p. in alum, and OVA i.n. to establish AHR in these mice. The mice were then vaccinated either with OVA-IL-18 DNA, OVA DNA, IL-18 DNA, OVA DNA + IL-18 DNA, or irrelevant DNA (as indicated in Fig. 5A), and AHR was measured after a final i.n. OVA boost. Mice that received irrelevant DNA developed strong AHR. In contrast, vaccination of the mice with the OVA-IL-18 DNA constructed greatly reduced AHR and was significantly more effective than OVA DNA alone (Fig. 5C). Vaccination with OVA-IL-18 DNA also reduced OVA-specific IL-4 production compared with the other DNA constructs (Fig. 5C). In addition, the OVA-IL-18 plasmid was much more effective than the OVA plasmid in reducing OVA-specific IgE production (OVA plasmid-treated group, 360 ± 800; OVA-IL-18 plasmid treated group, 2968 ± 81 ng/ml). These experiments demonstrated that OVA-IL-18 DNA, but not OVA DNA, could boost OVA-specific IFN-γ production and reduce IL-4 and IgE production, even when given in the context of ongoing AHR.

Discussion
In this study, we demonstrated that an OVA-IL-18 fusion DNA construct was highly effective in preventing and reversing allergen-induced AHR in a murine asthma model. Previous studies have demonstrated the usefulness of allergen DNA immunization in the prevention of allergic diseases and AHR (6, 12, 13, 35, 36), but allergen DNA vaccination has not been previously reported to successfully reverse ongoing AHR, a cardinal feature of asthma. We now describe an OVA-IL-18 DNA construct that effectively corrected established AHR in an allergen-specific fashion when administered only twice. Although both the OVA-IL-18 and the OVA DNA constructs, when administered to naive mice, prevented the subsequent induction of AHR and reduced allergen-specific IgE production, the OVA-IL-18 DNA was unique among the cDNA constructs in its capacity to reverse established AHR. The protective effects of OVA-IL-18 appeared to be mediated by IFN-γ and CD8 cells, and because the OVA plasmid was not capable of reversing established AHR, we conclude that the addition of IL-18 as a fusion construct greatly enhanced the immunogenicity and effectiveness of plasmid vaccination. These results indicate that the addition of IL-18 to allergen DNA constructs may substantially improve the immunogenicity and efficacy of allergen cDNA vaccines, and suggest that vaccination with allergen-IL-18 DNA construct greatly enhanced IFN-γ production, but the effect on IFN-γ production was confined to the OVA-specific response.

Inhibition of AHR depends upon CD8+ T cells and IFN-γ
To investigate the mechanisms by which vaccination with OVA-IL-18 affected OVA-specific responses, we administered blocking Ab to IFN-γ or Ab to CD8+ T cells during the immunization protocol. As expected, mice that received irrelevant DNA and control mAb developed strong AHR, which was significantly reduced in mice vaccinated with OVA-IL-18 DNA in the presence of control mAb (Fig. 4). Treatment with anti-CD8 mAb largely restored AHR in the OVA-IL-18 DNA-immunized animals. Anti-IFN-γ mAb also restored AHR, although to a lesser extent than that seen with CD8 depletion. Treatment of mice sensitized and challenged with OVA with either the anti-IFN-γ or the anti-CD8 mAb had no significant effect on AHR (data not shown). Thus, inhibition of AHR by OVA-IL-18 DNA was dependent upon both IFN-γ production and the presence of CD8+ cells.

Reversal of AHR by DNA vaccination
To determine whether DNA vaccination could reverse established AHR in addition to inhibiting the development of AHR, mice were first sensitized with OVA protein by administering OVA i.p. in alum, and OVA i.n. to establish AHR in these mice. The mice were then vaccinated either with OVA-IL-18 DNA, OVA DNA, IL-18 DNA, OVA DNA + IL-18 DNA, or irrelevant DNA (as indicated in Fig. 5A), and AHR was measured after a final i.n. OVA boost. Mice that received irrelevant DNA developed strong AHR. In contrast, vaccination of the mice with the OVA-IL-18 DNA construct greatly reduced AHR and was significantly more effective than OVA DNA + IL-18 DNA, IL-18 DNA alone, or OVA DNA alone. The reduction of AHR was consistent with the examination of BAL fluid, in which OVA-IL-18 DNA, but not OVA DNA, greatly reduced the percentage of eosinophils in BAL fluid (Fig. 5B). Eosinophils were still present in the lungs of mice treated with OVA-IL-18 DNA, perhaps consistent with the fact that some degree of AHR was present in these mice, and with the observation that IL-18 can recruit eosinophils into the airways (34). Nevertheless, these data demonstrate that 1) OVA-IL-18 cDNA but not OVA cDNA reverses ongoing AHR in previously sensitized mice; and 2) the activity of OVA-IL-18 DNA is clearly superior to that of OVA DNA in such sensitized animals.

IL-4 and IFN-γ measurements in OVA-immunized mice before and after DNA vaccination
Mice, vaccinated with DNA constructs after the establishment of OVA-induced AHR, were sacrificed and analyzed for cytokine production. Vaccination with OVA-IL-18 DNA resulted in a dramatic increase of IFN-γ production in bronchial LN cells as compared with that of animals receiving irrelevant DNA or OVA DNA alone (Fig. 5C). Vaccination with OVA-IL-18 DNA also reduced OVA-specific IL-4 production compared with the other DNA constructs (Fig. 5C). In addition, the OVA-IL-18 plasmid was much more effective than the OVA plasmid in reducing OVA-specific IgE production (OVA plasmid-treated group, 560 ± 800; OVA-IL-18 plasmid treated group, 2968 ± 81 ng/ml). These experiments demonstrated that OVA-IL-18 DNA, but not OVA DNA, could boost OVA-specific IFN-γ production and reduce IL-4 and IgE production, even when given in the context of ongoing AHR.
cDNA may be clinically effective in the treatment of patients with ongoing chronic allergic asthma.

The potent inhibitory effects of OVA-IL-18 DNA vaccination on AHR and IgE production was dependent on the fusion of the cytokine and allergen. Thus, vaccination with the OVA plasmid alone or with the IL-18 plasmid alone was less effective than the OVA-IL-18 fusion plasmid in inducing IFN-γ production, reducing IgE production, and preventing the development of AHR. In addition, codelivery of nonfused OVA DNA together with IL-18 DNA was much less effective compared with the fusion construct vector in reducing IgE production and preventing the development of AHR. This indicated that the presence of the IL-18 cDNA fused to the OVA cDNA was crucial for protection in this model, and was entirely consistent with our previous findings with OVA and IL-18 fusion proteins (28). IL-12, like IL-18, potently induces IFN-γ production and enhances Th1-biased immune responses, but IL-12 has significant Ag-nonspecific effects when administered in vivo. For example, in vivo administration of IL-12 has been associated in clinical trials with substantial morbidity and mortality (37), has been ineffective in suppressing Th2 recall responses (38, 39), and, when administered in high doses to mice, paradoxically enhances IgE synthesis (40). In contrast, we previously demonstrated that administration of the IL-12 when fused to OVA protein was associated with minimal Ag-nonspecific IL-12 effects and with maximized induction of IFN-γ production in an Ag-specific fashion. In addition, the fusion of cytokine and Ag has been shown to be effective in other models, for example, with idiotypic Ag of B cell lymphoma conjugated to GM-CSF, which enhanced the induction of protective immunity against a subsequent lethal tumor challenge (18, 19).

Our current studies suggest that the deleterious effects of IL-18 may be similarly minimized by fusion of IL-18 with OVA. Administration of high doses of IL-18 protein alone in vivo (7–20 μg/mouse, i.p.) appears to accentuate the undesirable effects of the cytokine, which include paradoxical increase in the recruitment of eosinophils into the airways (34), and enhanced allergic sensitization and IgE production (41). In contrast, we showed that vaccination with the fusion OVA-IL-18 cDNA construct, which more effectively reduced Th2-biased immune responses than did an OVA-IL-12 fusion cDNA construct (9), maximized the salutary IL-18 effects for asthma, presumably by focusing the activity of IL-18 onto OVA-specific T cells and B cells. These results, demonstrating protective effects of IL-18 on allergic inflammation, are consistent with the observation that IL-18 knockout mice developed increased allergen-induced eosinophilia (42). Moreover, administration of OVA-IL-18 as a plasmid rather than as protein may enhance the allergy-suppressive effects of IL-18 because the plasmid contains CpG motifs that induce the production of IL-12, which up-regulates IL-18 receptors on T cells (43) and synergistically down-modulates airway inflammation (44). The effects of IL-18 (and IL-12) may have been further augmented by fusion of the OVA and IL-18 in a plasmid because the OVA-IL-18 plasmid had the capacity to reverse established AHR, whereas administration of IL-18 and IL-12 together as proteins, did not (44). These

![FIGURE 4. Inhibition of AHR development by OVA-IL-18 DNA vaccination depends upon CD8+ cells and IFN-γ. Mice were immunized with the indicated plasmid DNA and sensitized to OVA as in Fig. 2A. To determine mechanism of protection, mice were injected repeatedly with mAb 53.6.7 (for CD8 depletion), XMG1.2 (for IFN-γ blocking), or LC4 (control mAb). One day after the last i.n. challenge with OVA, AHR was measured as in Fig. 2A. Data are expressed as mean percent above baseline ± SEM of five mice. Treatment with mAb to either CD8 or IFN-γ partially reversed the DNA-induced protection from AHR.](http://www.jimmunol.org/)

![FIGURE 5. A. Reversal of AHR by OVA-IL-18 DNA vaccination. To determine whether OVA-IL-18 DNA vaccination could reverse established AHR, mice were first sensitized to OVA (see Materials and Methods), and then immunized twice with either OVA DNA or OVA-IL-18 DNA (left) or OVA DNA, IL-18 DNA, OVA-IL-18 DNA, or a combination of OVA DNA + IL-18 DNA (right). AHR in response to methacholine challenge was measured as above. Only OVA-IL-18 DNA, and not OVA DNA alone, reversed established AHR under these conditions. Data are expressed as mean percent above baseline ± SEM of five mice. B. Recruitment of specific cell types into the airways of immunized mice. BAL was performed on the above mice just before sacrifice, and the percentages of eosinophils, lymphocytes, and macrophages (identified morphologically) were tabulated. Only OVA-IL-18 DNA vaccinated mice showed a decrease in eosinophil proportion, along with a rise in lymphocyte proportion of total BAL cells. Data are expressed as mean ± SD of five mice. C. IFN-γ and IL-4 secretion by bronchial LN cells from the vaccinated mice. Within 5 days of the last challenge, mice were sacrificed and bronchial LN cells were restimulated in vitro with OVA (100 μg/ml). IFN-γ and IL-4 levels in the culture supernatants were measured by ELISA. Only OVA-IL-18 DNA-immunized mice showed an appreciable increase in IFN-γ production and decrease in IL-4 production. Error bars represent SD of triplicate ELISA wells. All results are representative of two different experiments.](http://www.jimmunol.org/)
studies thus suggest that the strategy of delivering IL-18 conjugated with Ag as a plasmid vaccine may be applicable to treatment of allergic disease, particularly because the major allergens (and in many instances the major allergenic proteins) have been identified.

The inhibitory effect of OVA-IL-18 DNA on AHR was dependent on the presence of CD8+ T cells because the protective effects of OVA-IL-18 DNA could be almost completely reversed by depletion of CD8+ T cells. This observation supports other studies demonstrating the important role of CD8+ T cells in asthma. For example, Hsu et al. demonstrated that the protective effect of allergen DNA vaccination could be transferred with CD8+ T cells (12). Furthermore, animal experiments have revealed that CD8+ T cells regulate IgE production and allergen-induced AHR (45–48).

In our model, the induction of regulatory CD8 cells may have been enhanced by the potent capacity of IL-18 to induce CD8 T cells (49), and by the administration of OVA as cDNA, which may skew Ag presentation through an endogenous pathway. It is well established that peptides derived from intracellular Ags are generally presented to CD8+ T cells by MHC class I molecules (50), and this Ag presenting pathway may be important in the induction of regulatory CD8 cells when allergen cDNA is administered i.m.

The inhibitory effect of OVA-IL-18 DNA on AHR was also partially dependent on IFN-γ activity because coadministration of anti-IFN-γ mAb partially prevented the effects of OVA-IL-18 DNA. Both IL-18 as well as CpG motifs present on the vector 1

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